MOLECULAR SYSTEMATICS OF SUBTERRANEAN TERMITES *COPTOTERMES* SPP., MICROSATELLITE DEVELOPMENT AND POPULATION GENETICS OF *Coptotermes gestroi* (WASMANN) (BLATTODEA: RHINOTERMITIDAE)

YEAP BENG KEOK

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BY

YEAP BENG KEOK

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LIST OF ABBREVIATION

ANOVA	Analysis of variance
bp	Base pair (s)
°C	Degree Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
E	East
EDTA	Ethylene diaminetetraacetic acid
h	Hour (s)
$MgCl_2$	Magnesium Chloride
min	Minute (s)
N	North
NaCl	Sodium chloride
PCR	Polymerase chain reaction
P	Probability
SDS	Sodium Dodecyl Sulfate
sec	Second (s)
W	West

SISTEMATIK MOLEKUL ANAI-ANAI TANAH *COPTOTERMES* SPP., PERKEMBANGAN MIKROSATELIT DAN GENETIK POPULASI *Coptotermes gestroi* (WASMANN) (BLATTODEA: RHINOTERMITIDAE)

ABSTRAK

Disertasi ini memfokuskan kajian sistematik dan hubungan filogenetik antara anai-anai tanah (genus *Coptotermes*), di samping menentukan genetik populasi khususnya spesis *Coptotermes gestroi* (Wasmann). Hubungan filogenetik 14 spesis putatif *Coptotermes*: *Coptotermes acinaciformis* Froggatt, *Coptotermes cochlearus* Xia & He, *Coptotermes curvignathus* Holmgren, *Coptotermes dimorphus* Xia & He, *Coptotermes frenchi* Hill, *Coptotermes formosanus* Shiraki, *Coptotermes gestroi*, *Coptotermes guangzhouensis* Ping, *Coptotermes heimi* (Wasmann), *Coptotermes kalshoveni* Kemner, *Coptotermes lacteus* (Froggatt), *Coptotermes sepangensis* Krishna, *Coptotermes travians* (Haviland), dan *Coptotermes vastator* Light ditentukan dengan menggunakan jujukan DNA mitokondria (gen 12S,16S, dan COII). Jujukan sedia ada untuk spesies-spesies di atas yang diperoleh daripada GenBank turut disertakan dalam analisis ini. Analisis matrik nukelotida gabungan gen 12S, 16S dan COII menggunakan parsimoni maksimum (maximum parsimony) dan kemungkinan maksimum (maximum likelihood) menunjukan dua klad utama dengan enam klad sampingan: I (*C. acinaciformis*), II (*C. lacteus* dan *C. frenchi*), III (*C. curvignathus*), IV (*C. kalshoveni*, *C. sepangensis* dan *C. travians*), V (*C. gestroi*, *C. heimi*, dan *C. vastator*), dan VI (*C. formosanus*, *C. cochlearus*, *C. dimorphus* dan *C. guangzhouensis*). Berdasarkan model 2-parameter Kimura, jujukan interspesifik berpasangan antara *C. gestroi*, *C. vastator*, dan *C. heimi* menunjukkan perbezaan hanya sehingga 0.80% antara *C. gestroi* dan *C.*

vastator dan 2.13% antara *C. vastator* dan *C. heimi*. Sukatan morfometrik menggunakan 16 karakter menunjukkan banyak pertindihan di antara individu-individu yang dikaji daripada tiga spesies ini. Berdasarkan filogenetik molekular dan data morfometrik, adalah dicadangkan bahawa *C. vastator* dan *C. heimi* merupakan sinonim junior *C. gestroi*. Selain itu, turut dicadangkan bahawa *C. cochlearus* dan *C. dimorphus* berkemungkinan merupakan sinonim junior *C. formosanus* dengan perbezaan nukleotida sehingga 1.0%. Seterusnya, 11 penanda mikrosatelit dihasilkan untuk *C. gestroi*. Lapan daripada 11 penanda mikrosatelit tersebut digunakan untuk menentukan struktur koloni dan populasi serta kemungkinan mod organisasi reproduktif spesies *C. gestroi*. Sejumlah 89 koloni telah disampel daripada tujuh buah negara (Malaysia, Thailand, Singapura, Indonesia, Filipina, Taiwan, dan Hawaii) dan dibahagikan kepada 12 kumpulan populasi putative (Kedah, Pulau Pinang, Seberang Prai, Kuala Lumpur, Johor, Sabah, Singapore, Thailand, Indonesia, the Philipines, Taiwan, and Hawaii) mengikut lokasi geografinya. Analisis genotip pekerja anai-anai dalam koloni menunjukan 72 (80%) merupakan famili ringkas. 17 koloni lain (20%) merupakan famili kembangan yang diterajui banyak neotenik yang berkemungkinan berasal daripada pasangan reproduktif primer. Terdapat perbezaan genetik yang kurang ketara (F_{ST} = 0.073) antara populasi anai-anai semenanjung Malaysia (Kedah, Pulau Pinang, Seberang Prai, Kuala Lumpur, Johor), mencadangkan aliran genetik yang sederhana sesama populasi ini. Secara relatif, terdapat aliran genetik yang sederhana (F_{ST} = 0.192) di antara kesemua 12 populasi putatif yang dikaji, serta terdapat korelasi positif antara jarak genetik dan jarak geografi yang menyarankan aliran genetik yang terhad antara populasi tersebut. Kesimpulannya, tidak terdapat pengasingan jarak genetik antara dua populasi yang terbesar (Pulau

Pinang dan Kuala Lumpur) mungkin disebabkan oleh penglibatan manusia dalam penyebaran dan frakmentasi koloni-koloni di kawasan bandar.

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ABSTRACT

This dissertation focused on the systematics and phylogenetic relationships of the subterranean termites (genus *Coptotermes*), and population genetics of *Coptotermes gestroi* (Wasmann). The phylogenetic relationships of 14 putative *Coptotermes* spp. [*Coptotermes acinaciformis* Froggatt, *Coptotermes cochlearus* Xia & He, *Coptotermes curvignathus* Holmgren, *Coptotermes dimorphus* Xia & He, *Coptotermes frenchi* Hill, *Coptotermes formosanus* Shiraki, *Coptotermes gestroi*, *Coptotermes guangzhouensis* Ping, *Coptotermes heimi* (Wasmann), *Coptotermes kalshoveni* Kemner, *Coptotermes lacteus* (Froggatt), *Coptotermes sepangensis* Krishna, *Coptotermes travians* (Haviland), and *Coptotermes vastator* Light] were determined using mitochondrial DNA sequences (12S, 16S, and COII genes). Available sequences from the Genbank for these species were also included in the analyses. Maximum parsimony and maximum likelihood of the combined nucleotide matrices of the 12S, 16S, and COII genes resulted in two major clades with six subclades: I (*C. acinaciformis*), II (*C. lacteus* and *C. frenchi*), III (*C. curvignathus*), IV (*C. kalshoveni*, *C. sepangensis*, and *C. travians*), V (*C. gestroi*, *C. heimi*, and *C. vastator*), and VI (*C. formosanus*, *C. cochlearus*, *C. dimorphus*, and *C. guangzhouensis*). The interspecific pairwise sequence divergence based on the Kimura 2-parameter model among *C. gestroi*, *C. vastator*, and *C. heimi* was only 0.80% between

C. gestroi and *C. vastator* and 2.13% between *C. vastator* and *C. heimi*. Morphometric measurements of 16 characteristics revealed numerous overlaps among the examined individuals of these three species. Based on the molecular phylogenetics and morphometric data, it is proposed that *C. vastator* and *C. heimi* are junior synonyms of *C. gestroi*. In addition, *C. cochlearus* and *C. dimorphus* are possible junior synonyms of *C. formosanus* (with nucleotide differences of up to 1.0%).

Next, 11 microsatellite markers were developed for *C. gestroi*. Eight of the microsatellite markers were used to infer the colony and population structure and possible modes of reproductive organization of *C. gestroi*. A total of 89 colonies were sampled from seven countries (Malaysia, Thailand, Singapore, Indonesia, the Philippines, Taiwan, and Hawaii) and sorted into 12 putative population groups (Kedah, Penang Island, Penang mainland, Kuala Lumpur, Johor, Sabah, Singapore, Thailand, Indonesia, the Philipines, Taiwan, and Hawaii) based on their geographical localities.. Analysis of worker genotypes within colonies suggested that 72 colonies (80%) were simple families. The other 17 colonies (20%) were extended families headed by numerous neotenics, which probably descended from the primary pair of reproductives. There was negligible genetic differentiation (F_{ST} = 0.073) among the Peninsular Malaysia populations (Kedah, Penang Island, Penang mainland, Kuala Lumpur, and Johor population groups) suggesting moderate gene flow among them. Comparatively, there was moderate genetic differentiation (F_{ST} = 0.192) among the 12 putative studied population groups. In these colonies, a positive correlation between genetic distance and geographic distance was detected, suggesting rather restricted gene flow among them. Finally, there was no significant isolation by distance within the two largest colonies

(Penang and Kuala Lumpur); presumably humans aid in the dispersal and fragmentation of colonies in cities.

CHAPTER ONE

GENERAL INTRODUCTION

Termites are eusocial insects that live close together in large communities of several hundred to several million individuals. These communities are composed of functional reproductives together with numerous apterous sterile soldiers and workers, nymphs, and broods in a physically connected structure (nest) (Thorne *et al.* 1999). Ecologically, termites thrive in abundance. They are the primary insect group adapted to consume wood and dry plants by turning this nutritionally poor matter into protein biomass, and they provide a possible input of nitrogen through symbiont fixation (Wood and Sands 1978, Abe 1995). These insects also damage agricultural crops, wooden constructions, and other wood products in most subtropical and tropical countries, including Malaysia (Lee *et al.* 1999, Lee 2007).

Based on their colonial behaviour and food consumption, termites can be grouped into dry wood, damp wood, harvester, or subterranean termites (Nutting and Jones 1990). Dry wood termites can derive water metabolically and thus do not require moisture from their environment. They are usually found infesting structures above the soil surface, such as posts, stumps, and even wood on the 15th floors of commercial buildings (Krishna 1989, Robinson 1996). Damp wood termites require relatively high moisture in the wood they consume. They are found in dead, damp, and rotten logs (Krishna 1989). Harvester termites store food products, or harvest fungus which grows in their galleries (Collins 1981, Darlington 1982). Subterranean termites establish large colonies that are in contact with the soil (Krishna 1989). Of the 2600 termite species in the world, subterranean termites of the genus *Coptotermes* are by far the most

destructive to human carpentry endeavors, costing billions of dollars annually for damage repair and treatment (Su and Scheffrahn 1990, 2000). Once the soil nest is established, subterranean termites may enter unprotected wooden structures through contact with the soil, cracks in masonry, or by shelter tubes constructed from soil and glandular secretions.

Coptotermes spp. are believed to have originated in the Orient, from which they were transported around the world, probably as infestations in the hulls of wooden boats and in lumber. *Coptotermes* spp. appear to be environmentally adaptive across a wide and varied geography (Weesner 1970). In the United States and Hawaii, subterranean termite control and damage repair costs increased from US$ 2 billion to as high as US$ 11 billion annually in year 2002 (Jones 2000, Su 2002). In Southeast Asia, management efforts of termites cost an estimated US$ 400 million per year, and *Coptotermes* spp. are responsible for >90% of the total infestation (Lee 2002, 2007). With the gradual establishment of large-scale forest plantations, *Coptotermes* spp. became major pests of fast growing trees and cause a considerable amount of damage in plantations.

Coptotermes taxonomy is complex and has been plagued with controversies. Identification based on morphology is difficult and sometimes erratic because morphological traits are determined by both genetic and environmental influences (e.g., climate and diet) (Kirton 2005). Differences can be great between genera, but not between species (Krishna 1970). Errors often have been made in both naming and identifying *Coptotermes* spp.. For example, inconsistencies in the pest status of *Coptotermes havilandi* (Holmgren) in different regions of its geographic range were due to misidentification and taxonomic confusion between *Coptotermes travians* (Haviland),

C. havilandi, and *Coptotermes gestroi* (Wasmann) (Kirton and Brown 2003). Kirton and Brown (2003) also suggested that *Coptotermes heimi* (Wasmann) might be a junior synonym of *C. gestroi*. Kirton (2005) suspected that *Coptotermes vastator* Light was a junior synonym of *C. gestroi*.

Molecular phylogenetic analyses can reveal the relationships among populations and differentiate species regardless of the termite caste (Szalanski *et al.* 2003). Many recent studies have demonstrated the potential of using mitochondrial gene sequence analysis for accurate species identification and to study phylogeny and gene flow (Liu and Beckenbach 1992, Kambhampati 1995, Kambhampati *et al.* 1996, Frati *et al.* 1997, Miura *et al.* 1998, Austin *et al.* 2004, Ye *et al.* 2004, Jenkins *et al.* 2007).

Kirton (2005) reviewed the importance of accurate identification to the pest management industry. With the recognition of a single pest species, information about the species from different geographical regions can be pooled. Industry will also be able to avoid duplicative testing of termite management strategies for what was originally thought to be different pest species in different regions, which will in the long run save time, money and resources. Thus, the objectives of this thesis were:

1. To resolve the genetic relationships among *C. vastator*, *C. heimi*, and *C. gestroi*;
2. To study the molecular systematics of *Coptotermes* spp. from East Asia via phylogenetic analyses of three mitochondrial genes (12S, 16S, and COII);
3. To isolate microsatellite DNA loci from *C. gestroi* for population studies;
4. To characterize the colony and genetic structure of *C. gestroi* populations from Asia.

CHAPTER TWO
LITERATURE REVIEW

2.1 Significance of termites in the ecosystem

The fossil record indicates that termites evolved about 220 million years ago (Collins 1988, Thorne and Carpenter 1992). Evidence indicates that phylogenetically termites are closely related to cockroaches (Kambhampati 1995). Termites are thought to be derived from a primitive group of wood-feeding cockroaches (*Cryptocercus*). Both have unique mutualistic intestinal protists, endosymbiotic flavobacteria in the visceral section of the fat body, and in some higher forms, externally cultivated basidiomycete fungi (Bignell and Eggleton 1998, Nalepa and Bandi 2000).

Termites fall into seven families consisting of 281 genera with over 2600 described species (Krishna 1970, Kambhampati and Eggleton 2000). The majority of termites live in tropical and subtropical regions, but they also spread into the temperate zone. About two-thirds of the Earth's land surface between the latitudes 48° N and 45° S is inhabited by termites (Lee and Wood 1971). In tropical and subtropical regions, their numbers exceed 6000 individuals m^{-2}, and their biomass ranges from 5 and 50 gm^{-2}, often surpassing biomass of mammalian herbivores, which range from 0.01 and 17.5 g m^{-2} (Lee and Wood 1971, Collins 1989).

Termites can be classified into phylogenetically lower and higher groups (Krishna 1970) (Figure 2.1). The lower termite group consists of six families. They harbor a dense and diverse population of both prokaryotes and flagellated protists in their gut. In terms of their diet, they are restricted to wood or grass (Honigberg 1970, Noirot 1992). Higher termites (Termitidae) include soil-feeding (humivorous), wood and

grass-feeding (xylophagous), and fungus-cultivating species. The majority of termite species belong to the higher termite group (80% of all species). These species also harbor a dense and diverse array of prokaryotes but typically lack flagellated protists and have a more elaborate morphology and social organization (Honigberg 1970, Noirot 1992). Instead, an acquisition of cellulases with the food (in case of the fungus-cultivating termites) or a host origin of the cellulolytic activities has been suggested (Breznak and Brune 1994). It is widely accepted that termites have a major impact on the decomposition of plant material, thereby increasing microsite heterogeneity, humification, soil conditioning (i.e., translocating and altering soils physically and chemically and maintaining soil fertility), the release of immobilized N and P, the improvement of drainage and aeration, and an increase in exchangeable cations (Lee and Wood 1971, Wood and Sands 1978, Wood 1988, Collins 1989, Martius 1994, Brussaard and Juma 1996, Lavelle *et al.* 1997, Holt and Lepage 2000, Donovan *et al.* 2001). Therefore, termites actively take part in the permanent alteration of their habitat, especially in tropical ecosystems.

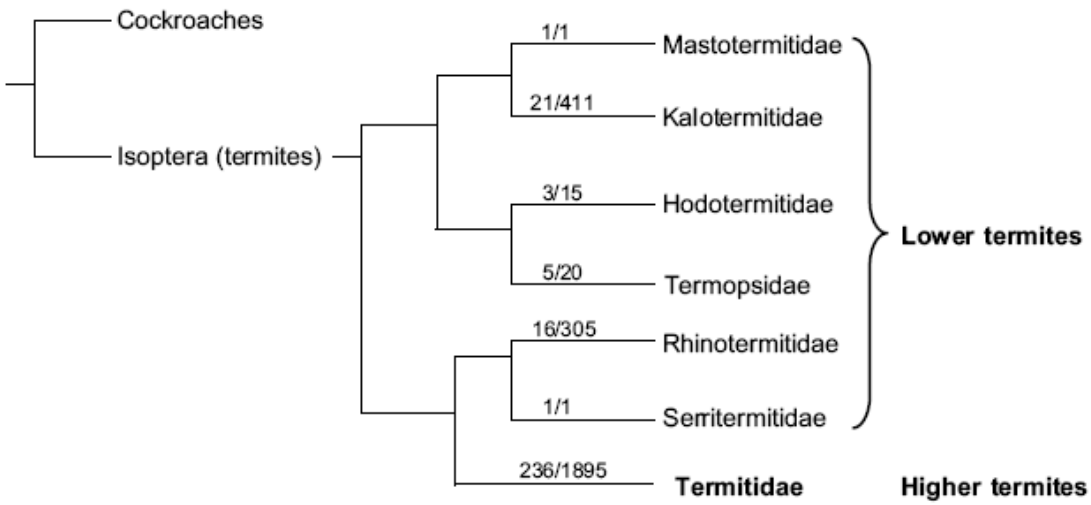

Figure 2.1: Phylogenetic scheme of termite evolution showing the presumed relationships of the seven termite families (adapted from Higashi and Abe 1997). The numbers on the lines represent the number of genera/species in the different families (Abe *et al.* 2000).

Due to their high biomass and density, termites significantly contribute to the emission of atmospheric trace gases such as methane and carbon dioxide (Zinder 1993). Zimmerman *et al.* (1982) estimated the contribution of termites to global methane emissions to be as high as 45%. However, more recent estimations based on a larger data set, together with consideration of the great differences in methane emission rates among different termite species, reduced their contribution to less than 5% (Sanderson 1996, Bignell *et al.* 1997, Sugimoto *et al.* 1998).

2.2 Subterranean termites: *Coptotermes* spp.

The Rhinotermitidae evolved from an extinct ancestral Hodotermitidae, which shared the primitive hodotermitid imago-worker mandibles of the Archotermopsis-Stolotermes type, with three marginal teeth in the left mandible, and also possessed

ocelli (Ahmad 1950). Emerson (1955) speculated that the Oriental region was the main center of origin and dispersal of the Rhinotermitidae. This is the most important family of lower termites in Malaysian forests (Collins 1988, Tho 1992). They occur in standing or fallen trunks and limbs and can cause severe damage to timber and living trees. The Rhinotermitidae includes six subfamilies: Coptotermitinae, Heterotermitinae, Psammotermitinae, Termitogetoninae, Stylotermitinae, and Rhinotermitinae.

The Coptotemitinae, includes a single genus *Coptotermes*, is the most primitive Rhinotermitidae subfamily, with 71 described species (Kambhampati and Eggleton 2000). *Coptotermes* contains the largest number of species that are economically important structural pests (Su and Scheffrahn 2000). *Coptotermes* spp. are widely distributed in tropical and subtropical regions (Figure 2.2), and their spread is facilitated by humans. Environmental factors are known to affect the geographical distribution of subterranean termites (Kofoid 1934). Temperature and moisture are the main environmental factors that affect both geographical and local distribution of termite genera and species (Kofoid 1934). Critical temperatures define ecological or behavioral tolerance (Hu and Appel 2004), and temperature changes may influence seasonal, local, and diurnal distribution. Local occurrence of termites is determined by moisture (atmospheric and soil), temperature (which regulates relative humidity), and moisture (which accelerates, restricts, or inhibits vital processes in termites) (Kofoid 1934).

Subterranean termites require a highly humid environment, and this requirement may limit their mobility. However, when they invade tubs or pots containing plants growing in soil, timber that is kept at a constant high moisture level, or timber in contact with the ground for some time before shipment, *Coptotermes* spp. are readily transported by humans (Adamson 1938, Lever 1952). Termites can build nests out of packing

material and fill aboveground voids, which can retain moisture; these abilities are likely to enhance the survival of these pests when they are inadvertently transported (Jenkins *et al.* 2002).

Many *Coptotermes* spp. have been identified outside of their native range, including *Coptotermes acinaciformis* (Froggatt), *Coptotermes amanii* (Sjöstedt), *Coptotermes crassus* Snyder, *Coptotermes formosanus* Shiraki, *Coptotermes frenchi* Hill, *C. gestroi* , *C. heimi*, *Coptotermes lacteus* (Froggatt), *Coptotermes testaceus* (Linnaeus), and *C. vastator*. Of these species, *C. formosanus* and *C. gestroi* are the most invasive species. They often become economically important pests when they become established in new regions. In the United States, subterranean termite control and damage repair cost approximately US$ 11 billion per year (Su 2002). In Southeast Asia, management of subterranean termites costs an estimated US$ 400 million per year, and *Coptotermes* spp. are responsible for >90% of the total infestation (Lee 2002, 2007).

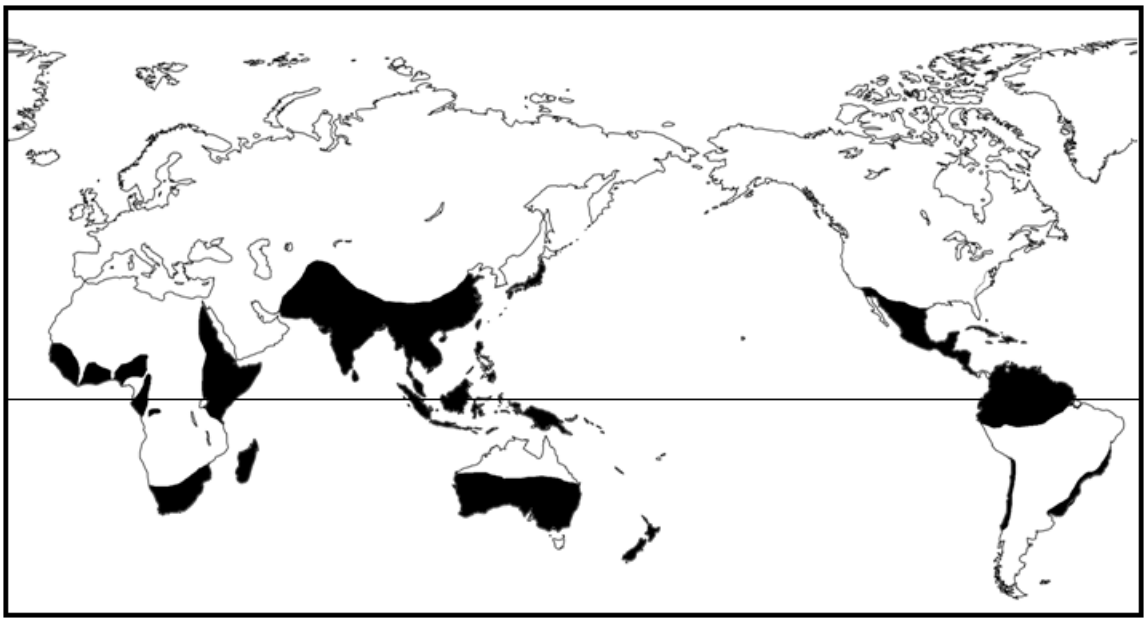

Figure 2.2: Distribution of *Coptotermes* spp. (dark area). Modified from Pearce (1997).

2.2.1 Colony structure

Colony founding in subterranean termites is believed to begin after a mating flight, when a male and female alate pair begin reproducing and establish a colony (Thorne *et al.* 1999). Over time, neotenics (non-alate derived reproductives) may replace the loss of one or both of the primary reproductives within the nest. A few or dozens of neotenics may develop in colonies, and then inbreeding is believed to occur. This is a trait unique to some families of termites, including the Rhinotermitidae, and it should increase the genetic relatedness within colonies and the genetic contrast among colonies.

According to Myles (1999), nymphoid and ergatoid neotenics in the Rhinotermitidae may serve either as replacement reproductives or as supplementary reproductives. The following species of the genus *Coptotermes* are known to produce neotenic reproductives: *C. acinaciformis, C. amanii, C. curvignathus, C. formosanus, C. frenchi, C. heimi, C. intermedius, C. lacteus, C. niger, C. sjostedt*, and *C. vastator* (Lenz

and Barrett 1982, Lenz *et al.* 1986, Lenz *et al.* 1988, Lenz and Runko 1993, Myles 1999). The rate of neotenic development varies greatly among *Coptotermes* spp. (Lenz *et al.* 1988). Costa-Leonardo *et al.* (1999) reported that *C. gestroi* produces nymphs throughout the year and non-functional neotenics, even in the presence of the imaginal pair. Functional neotenics were also found in colonies of this species, but physogastry was less developed than that of the primary queens (Lelis 1999).

Besides swarming, termite colony proliferation by "budding" has also been suggested based on observational and empirical studies (Thorne *et al.* 1999). As colonies grow and expand their foraging range, groups of workers (foragers) can split off from the main colony and establish their own independent colony. This creates complicated colony structures consisting of widespread interconnected foraging areas and multiple nests containing a variable number of reproductives. Budding may be detected by the presence of population viscosity (Husseneder *et al.* 1998, Vargo 2003b). It is also possible for different colonies to fuse or for unrelated reproductives to be adopted into colonies (Clément 1986, Jenkins *et al.* 1999, Matsuura and Nishida 2001); these processes would lower the genetic contrast among colonies and decrease genetic relatedness within colonies.

The subterranean termite nest in its simplest form consists simply of galleries that extend into the ground and form a more or less diffuse network, with enlarged and more spacious chambers in some places. Such diffuse structures do not have any sharp boundaries, and the network of chambers and galleries also varies over time. All phases of intermediate structure exist between the diffuse nest and the concentrated nests, which occupy a definitive volume and are very distinct from the surrounding soil (Noirot 1970).

Concentrated nests most often are constructed as a single unit, but sometimes they consist of several independent units that have the same architecture and are connected by subterranean galleries. Each unit is termed a calie, and when a group of calies are associated with a single colony, the nest is termed polycalic. In the Rhinotermitidae, certain *Coptotermes* spp. may build concentrated subterranean nests composed of carton which is probably stercoral (Noirot 1970).

A colony of subterranean termites consists of functional reproductives (king and queen), soldiers, workers, nymphs and brood that live together in close association and forming a physically connected structure (nest) (Krishna 1989). The main role of the queen is to produce eggs. The queen develops an enlarged abdomen containing ovariales and associated tissues (a condition known as being physogastric) (Collins 1984). Besides laying eggs, the queen also is involved in pheromonal regulation of the production of each caste in a colony (Noirot and Noirot-Timothee 1969). Large colonies may include a number of supplementary reproductives, which produce eggs to augment or replace the founding queen (Bignell and Eggleton 1998).

Soldiers and workers are wingless and can be either sterile male or female (Weesner 1987). They lack compound eyes and ocelli (Ross 1956, Thorne 1996, Pearce 1997). Soldiers usually represent one-tenth of the population at most (Harris, 1957). The major role of soldiers is to defend the colony (Bignell and Eggleton 1998). Thus, they are morphologically large in size and have enlarged mandibles or stopper-like heads for defensive purposes (Krishna 1989). In certain genera, such as *Coptotermes*, soldiers have a frontal gland that discharges a defensive secretion through a frontal pore that can be toxic or repellent to intruders (Richards and Davies 1978). Soldiers are not capable of feeding on their own and thus depend on workers to feed them via trophallaxis. The

worker caste is the most numerous and plays a major role in the survival of the colony. They forage for food, which consists mainly of wood, paper, and other cellulose-based materials. They carry the food back to their nest, process the digesta, and feed other castes members (Thorne 1996). They are also responsible for constructing the mound and mud tubes (Harris 1957).

Winged reproductives (or alates) of both sexes are produced in large numbers in a mature colony (i.e., aged 3–4 years old) (O'Toole 2003). These alates are potential founders of new colonies when they swarm out of mature nests at particular times of the year (often during or just before rains) (Pearce 1997, Bignell and Eggleton, 1998, Lee and Robinson 2001). They make short, often rather feeble, dispersal flights and then pair up on the ground after the wings have been shed (dealation) (Bignell and Eggleton, 1998). The paired termites will then select a new nesting site, and once they are established mating takes place.

Like cockroaches, termites are hemimetabolic, meaning that they have egg, nymph, and adult stages but no pupal stage. The first batch of eggs is produced by the female within a few days after mating. The larvae are fed with nutrient-rich salivary secretions produced by the parents. The larvae normally undergo a number of moults until they achieve the mature form as sterile workers or soldiers, depending on the need of the colony (Harris 1957). Caste differentiation in termites is not genetically linked but rather is determined by extrinsic factors such as pheromones and hormones (Krishna 1970, Robinson 1996). When a colony is first founded, usually all of the larvae become workers; after some period of time, an occasional larva grows into a soldier (Harris 1957). The colony grows slowly for many years, accompanied by a continuous increase in the number of individuals, enlargement of the nest, and much building activity

(Bignell and Eggleton, 1998). Once the colony is well organized, larvae develop into winged termites and the full cycle of development is complete (Harris 1957).

2.2.2 Species identification, and distribution of *C. gestroi*

Coptotermes gestroi belongs to the family Rhinotermitidae and the genus *Coptotermes*. The head of *C. gestroi* soldiers is pear shaped, rounded laterally, and armed with slender and slightly incurved mandibles. No teeth are apparent on the mandibles. The head possesses a distinct pair of pale, crescent-shaped spots just antero-dorsal of the ocelli (Kirton and Brown 2003). Soldiers have a large opening on the forehead called the fontanelle, from the base of which one pair of setae project dorsal-laterally. The lateral profile of the top of the head just behind the fontanelle shows a weak bulge. The maximum head width of soldiers ranges from 1.34 to 1.53 mm, and the colour ranges from yellow to orange (Tho 1992). *Coptotermes gestroi* soldiers constitute about 10–15% of foraging groups, aggressively bite when challenged, and exude a white mucous-like secretion from the fontanelle (Scheffrahn and Su 2000). This secretion is used for defensive purposes, and it also gives the soldier body its white coloration (Tho and Kirton 1990, Chey 1996, Woods 2005). The head, pronotum, and dorsal abdomen are dark brown in *C. gestroi* alates. The darker pigmentation of the *C. gestroi* alate head provides a contrasting background for two light patches on the face called antennal spots. The alates of *C. gestroi* have a total length with wings of about 13–14 mm and a maximum head width of 1.4 mm.

Figure 2.3 shows the important morphological characters of the soldier caste that are commonly used in termite identification. Although still commonly used, many researchers believe that the traditional morphometric approach (i.e., a univariate method)

is inadequate for discriminating termite species because the key measurements usually overlap extensively. The multivariate morphometric approach using statistical analyses such as principal component analysis (PCA) and discriminant function analysis (DFA) seem to be better tools for termite identification (Cadrin 2000). In PCA, a relatively small number of factors that represent correlation among sets of many interrelated variables are identified. PCA does not require a *priori* grouping but rather combines and summarizes the associated measured variables into a number of principle components (PCs), which are referred to as patterns of covariation in size and shape in morphometric analysis (Wiley 1981).

DFA is a multivariate extension of analysis of variance (ANOVA) that can be used to study variation and covariation among taxonomic groups (Fisher 1936). It is an optimization technique used to categorize the sample according to the examined morphological variables. DFA requires a *priori* grouping, and discriminant functions (DFs) are calculated by deriving a weighted combination of variables that have maximal variances between groups relative to within groups. These DFs will reclassify the individuals into the designated groups (Turan 1999).

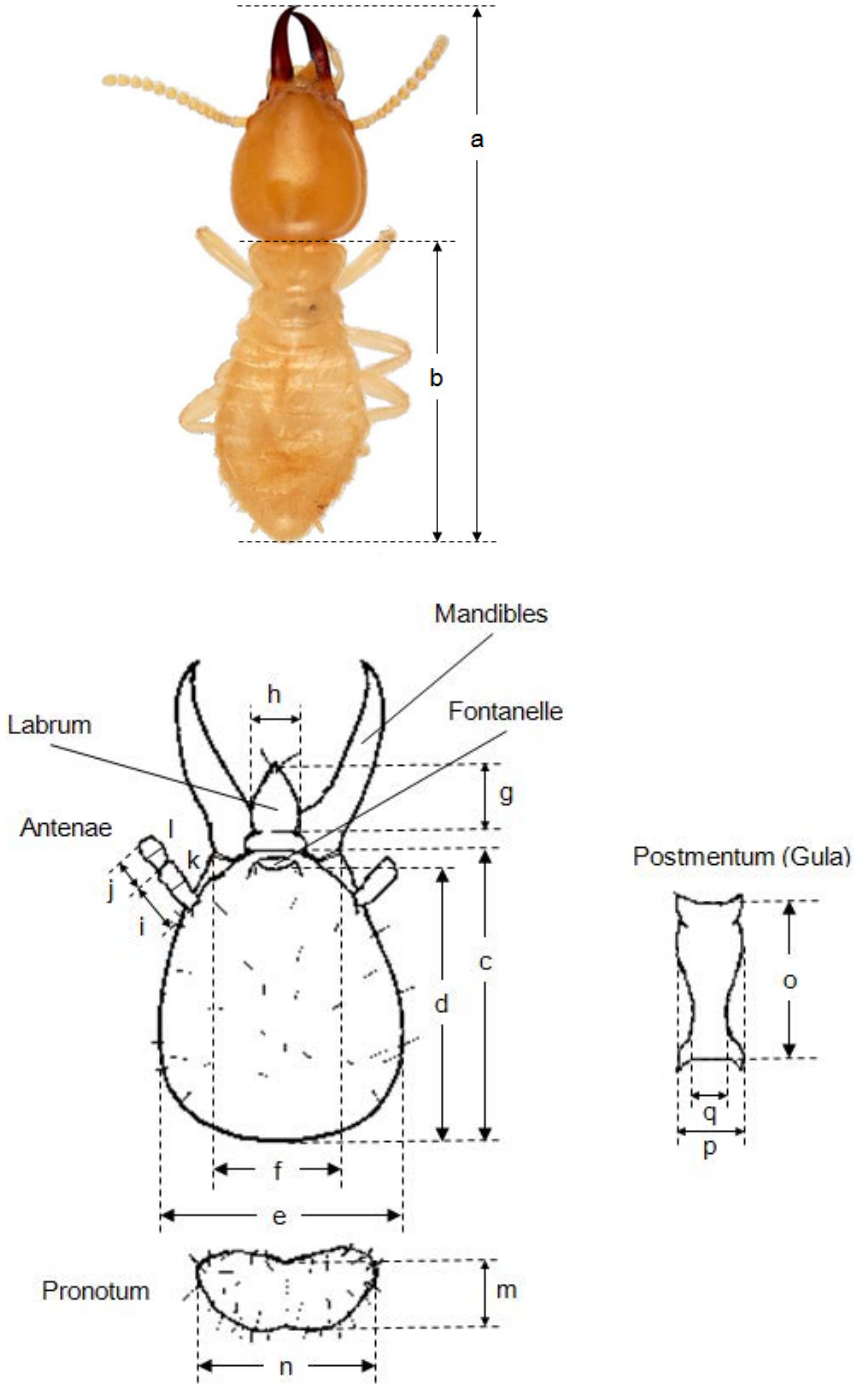

Figure 2.3: *Coptotermes gestroi* soldier, with a dorsal and profile view of the head, pronotum, and postmentum showing the morphological characteristics: (a) total length, (b) length without head, (c) length of head at base of mandibles, (d) head, length to fontanelle, (e) maximum width of head, (f) width of head at base of mandibles, (g) labrum length, (h) labrum maximum width, (i) segment I of antennae, length, (j) segment II of antennae, length, (k) segment I of antennae, width, (l) segment II of antennae, width, (m) pronotum length, (n) pronotum width, (o) gula length, (p) gula maximum width, (q) gula minimum width.

Coptotermes gestroi is an Asian subterranean termite that is endemic to Southeast Asia. It is the primary pest species of *Coptotermes* originating from the Indo-Malayan Region (Assam, Burma, Thailand, Malaysia, and the Indonesian archipelago) (Roonwal 1970, Tho 1992, Kirton and Brown 2003). Over the last century, human activity has spread this termite species far beyond its native range. It was collected in the Marquesas Islands (Pacific Ocean) in 1932 and in Mauritius and Reunion (Indian Ocean) in 1936 and 1957, respectively (Light 1932, Moutia 1936, Paulian 1957). *Coptetermes gestroi* has also been found in Madagascar (southern Indian Ocean) (Edwards and Mill 1986).

Coptotermes gestroi is limited to more tropical localities (to 26 ° N). In the New World tropics, this species was first reported in Brazil in 1923 and in Barbados in 1937. Recent collections from other West Indian islands include Antigua, Barbuda, Cuba, Grand Cayman, Grand Turk, Jamaica (Montego Bay and Port Antonio), Little Cayman, Montserrat, Nevis, Providenciales, Puerto Rico (San Juan), St. Kitts, and on a boat from the U. S. Virgin Islands (Araujo 1958, Constantino 1998, Scheffrahn *et al.* 1990, 2003). It has also been collected in southern Mexico.

In 1996, *C. gestroi* was collected for the first time in the continental United States from a storefront and a church in Miami, Florida (Scheffrahn *et al.* 1994, Su *et al.* 1997). In 1999, a colony of *C. gestroi* was discovered infesting a waterfront house in Key West, Florida. In 2001, a single alate was collected in Lauderhill, Broward Country, Florida. In 2004, it was verified that six structures in Key West were infested with *C. gestroi*. A boat moored off Fleming Key and another in dry dock on Stock Island (east of Key West) also were infested. Three private boats in Florida were found to have shipboard infestations: one arriving from Jamaica, one from Virgin Gorda, and one from

Providenciales. These boats were docked in Ft. Pierce, Hollywood, and Ft. Lauderdale, respectively.

In 2005, *C. gestroi* was found in the cities of Ft. Lauderdale and Riviera Beach (adjacent to the north of West Palm Beach). These discoveries are new records for established, land-based colonies of this species in Palm Beach and Broward Counties. The Riviera Beach infestations mark a substantial northward range expansion for this species in Florida, and they are the northernmost established colonies of this tropical species worldwide. More recently, *C. gestroi* was recorded for the first time in Taiwan (Tsai and Chen 2003).

2.2.3 Economic importance of *C. gestroi* in Southeast Asia

Coptotermes gestroi is one of the most economically important and widespread urban pests in Southeast Asia (Su *et al.* 1997, Kirton and Brown 2003, Lee *et al.* 2003, Kirton 2005, Kirton and Azmi 2005). It is a destructive pest of structural wood and agricultural crops in Thailand, Malaysia, and Indonesia (Ahmad 1965, Gay 1967, Roonwal 1979). Records show that *C. gestroi* in Southeast Asia attacks dead and dying trees of various species, construction timber, furniture, structural wood, plastics, and synthetic fibers (Roonwal 1979). *Coptotermes gestroi* forms large colonies, can penetrate a variety of materials and consume a wide range of wood types, and is always a severe structural pest wherever it occurs.

Termite damage and control is estimated to cost approximately US$ 400 million per year in Southeast Asia, and a large proportion of this is caused by *C. gestroi* (Lee 2007). In Malaysia and Singapore, 85% of buildings infested by termites in urban areas

are infested with *C. gestroi* (Kirton and Azmi 2005). In Thailand, 90% of termite infestations in urban areas are caused by *C. gestroi* (Sornnuwat *et al.* 1996).

2.3 Advances in molecular genetic techniques

The advent of molecular genetic approaches has provided a powerful method to gain insight into termite autecology and synecology. Termites are uncontroversially placed within the Orthopteroid group of insect orders (Boudereaux 1979, Hennig 1981). However, the relationships of termites, mantids, and cockroaches within the superorder Dictyoptera have been vigorously debated. Recent molecular phylogenetic studies that reviewed the position of Isoptera among the Dictyoptera concluded that termites are nested within cockroaches (Maekawa *et al.* 1999, Lo *et al.* 2000, 2003, Terry and Whiting 2005), thus making Blattaria paraphyletic as presently constituted. Eggleton (2001) agreed that termites probably are eusocial cockroaches; although their exact position has not yet been firmly established, *Cryptocercus* is the most plausible sister group. With a more comprehensive molecular phylogenetic study, Inward *et al.* (2007) proposed that the species presently included in Isoptera should be classified within the family Termitidae as part of the order Blattodea within the superoder Dictyoptera.

Others studies have focused on the family group relationships within the Isoptera (Noirot 1995, Kambhampati and Eggleton 2000, Thompson *et al.* 2000), species-level relationships within genera (Miura *et al.* 2000), and smaller scale phlyogenetic patterns, generally as part of taxonomic revision (Miller 1991, Costantino 1995, Roisin *et al.* 1996). Scheffrahn *et al.* (2005) provided genetic evidence for the synonymy of *Nasutitermes corniger*, *N. costalis*, and *N. polygynus*, and Austin *et al.* (2005) reported the synonymy of *Reticulitermes flavipes* and *R. santonensis*.

Besides systematics and taxonomic studies, techniques of molecular biology also provide important new insights into the development and caste differentiation, breeding structure, as well as colony and population dynamics (Roisin and Lenz 1999, Thorne *et al.* 1999, Lain´e and Wright 2003, Vargo 2003a, b, Raina *et al.* 2004).

2.3.1 Genetic markers

Various genetic markers, such as RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), RAPD (random amplification of polymorphic DNA), VNTR (variable number tandem repeat), microsatellite polymorphism, SNP (single nucleotide polymorphism), STR (short tandem repeat), mitochondrial DNA, allozymes, and DNA fingerprints, can be used to infer historical attributes of populations or species and to study evolutionary biology (e.g., phylogenetics and population genetics). Different genetic markers have different characteristics and inheritance patterns and therefore target different but overlapping research areas.

Table 2.1 shows some of the techniques that are useful for studying a vast range of issues. Although allozyme polymorphism has been successfully applied in studies of termites (Clément 1981), this biochemical technique requires either living samples or samples preserved by freezing at –80°C, and all samples have to be from the same caste. In general, DNA-based molecular methods are more useful. DNA sequences, which can be obtained rapidly by the polymerase chain reaction (PCR) and PCR-based cycle sequencing, are expected to be similar among different castes and developmental stages. Thus, termite samples from different castes can be preserved in ethanol, facilitating the

Table 2.1. Methods available for genetically characterizing individuals and populations and their applicability to each issue. Techniques with + can be used for the purpose specified, and several +s indicate that the technique has high utility; ? indicates that the technique is useful in only some cases; and – indicates that the technique is not useful in this context (Adapted from Frankham *et al.* 2002)

Issue	Chromosomes	Allozymes	mtDNA	RAPD	Microsatellites	DNA fingerprint
Non-instrusive sampling	–	–	+++	++	+++	-
Forensics	–	+	+++	++	++	++
Population size	–	–	+++	+	+	?
Estimating N_e	–	++	++	–	+++	?
Demographic history	–	–	++	–	+	?
Detecting and dating bottlenecks	–	++	++	++	+++	?
Detecting selection	+	+	+++	+	+++	++
Migration and gene flow	–	++	+	++	+++	++
Individual identification and tracking	–	–	++	+	+++	-
Population structure	–	++	+?	++	+++	++
Phylogeography	–	–	+++	–	+++	-
Source populations to recover endanger species	–	++	+	++	+++	+++
Introgression	+	++	+	++	+++	++
Secondary contact	–	–	+++	–	+++	+
Taxonomic status	+++	++	++	+++	+++	+++
Sites for reintroduction	–	–	+	+	+++	-
Populations for reintroduction	–	++	+	++	+++	+++
Reproductive systems	–	++	–	+	+++	?
Paternity	–	+	–	+	+++	+++
Founder relationships	–	?	–	+++	++	+++
Sources for new founders for endangered populations	–	++	+	++	+++	++

collection of samples. DNA sequence data contain appropriate phylogenetic information, which should alleviate the problem of homology assignment and incongruence.

2.3.2 Application of mitochondrial DNA for studying termite genetics

In the last 20 years, the extensive use of DNA sequence data and advances in analytical methods have transformed phylogenetic systematics. Phylogenetic relationships among termites, cockroaches, and mantises were examined based on mitochondrial ribosomal RNA genes (Kambhampati 1995, 1996). In 1996, the first termite molecular phylogeny using the mitochondrial 16S rRNA gene was published (Kambhampati *et al.* 1996). It is now universally accepted that DNA sequences are a rich source of characters for estimating phylogenies (Hillis *et al.* 1996).

Mitochondrial DNA (mtDNA) is a double-stranded ring of extrachromosomal DNA located in the mitochondria. It was discovered in the 1960s by electron microscopy as DNase-sensitive threads inside mitochondria and also by biochemical assays of highly purified mitochondrial fractions (Nass and Nass 1963, Haslbrunner *et al.* 1964). In most animals, mtDNA is a single circular molecule that contains a homologous set of genes encoding 13 proteins, two ribosomal RNA genes (rRNA), 22 transfer RNA genes (tRNA), and a non-coding section known as the D-loop or control region, and it ~16–20 kb in size (Figure 2.4).

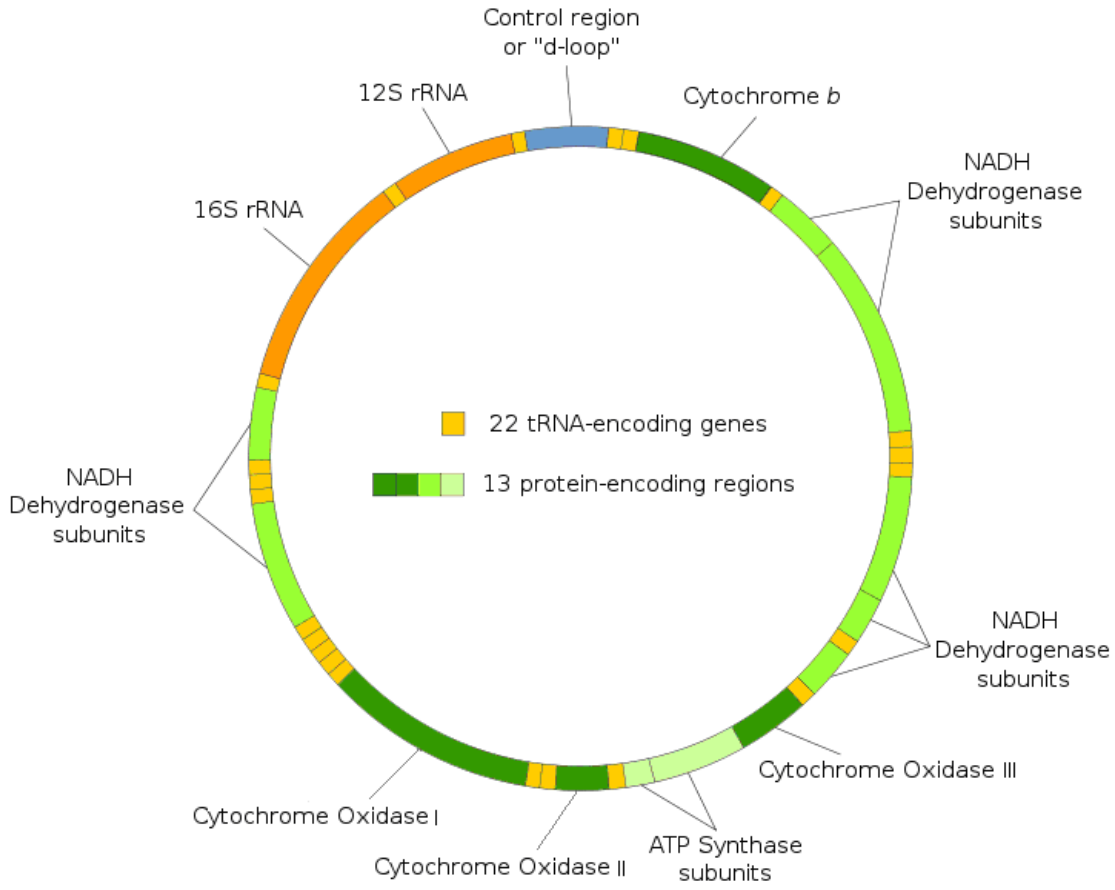

Figure 2.4: The genetic map of mtDNA. Modified from Crozier and Crozier (1993).

MtDNA is an ideal genetic marker, and it has been widely used in systematics and phylogenetic studies of termites (Liu and Beckenbach 1992, Kambhampati 1995, Kambhampati *et al.* 1996, Frati *et al.* 1997, Miura *et al.* 1998, Austin *et al.* 2004, Ye *et al.* 2004, Jenkins *et al.* 2007). Szalanski *et al.* (2003) reported the first molecular technique for *Reticulitermes* spp. differentiation in which >97% and 87% of the samples were successfully identified to species using 16S and COII mtDNA genes, respectively. Mitochondrial genes are known to evolve more rapidly than nuclear genes. The relatively fast mutation rate results in significant variation in mtDNA sequences between species and, comparatively, small variance within species. Differences between

haplotypes can be detected through length heterogeneity, restriction fragment analysis, or sequencing of mtDNA regions. Thus, mtDNA is a good marker for analyzing relatively close relationships, such as species relationships within a genus, and for population studies (Miura *et al.* 2000).

mtDNA is maternally inherited without recombination, thus making it suitable for identifying the distribution of matrilines within populations and colonies. Compared with nuclear DNA, mtDNA has a greater number of copies per cell, which increases the chance of obtaining a useful sample, especially from old samples that have been stored for a long time. In addition, mtDNA can be easily amplified from a variety of taxa and, because it is haploid, the sequence can be obtained without cloning. The high evolutionary rate and an effective population size of mtDNA provide the opportunity to uncover the pattern and tempo of recent historical events without an extensive sequencing effort. Finally, as an area of low recombination, the whole molecule can be assumed to have the same genealogical history. Therefore, mtDNA is the marker of choice in many population, biogeographic, and phylogenetic studies. Its use has been recommended in taxonomic studies, and Hebert *et al.* (2003) proposed that all described species be given an mtDNA sequence tag or bar-code.

More specifically, sequences of the mitochondrial genes cytochrome oxidase subunit II (COII), rRNA large subunit (16S), and rRNA small subunit (12S) have been extensively applied in phylogenetic reconstructions for a variety of taxa (Liu and Beckenbach 1992, Kambhampati 1995, 1996, Kambhampati *et al.* 1996, Frati *et al.* 1997, Miura *et al.* 1998, Austin *et al.* 2004, Ye *et al.* 2004, Jenkins *et al.* 2007). They are suitable for comparing closely related species and genera, and they have shown

consistent results in termites samples collected from different locations (Austin *et al.* 2005).

2.3.3 Application of DNA microsatellites for studying termite population genetics

Microsatellite loci were discovered in the late 1980s. Microsatellites are small sequences of 1–6 bp that are tandemly repeated and typically span between twenty to a few hundred bases and are widely dispersed in eukaryotic and prokaryotic genomes. (Beckmann and Weber 1992, Weber and Wong 1993, Hancock 1999, Schlötterer 2000). Microsatellites are commonly located in non-coding intergenic regions and demonstrate high levels of polymorphism (Litt and Luty 1989, Tautz 1989). The variability in the number of repeated nucleotides in an allele is caused by two potential mutational mechanisms: unequal crossing-over (UCO) also called gene conversion (Smith, 1976, Jeffrey *et al.*, 1994) and slipped-strand mispairing (SSM, also referred to as DNA polymerase slippage) (Levinson and Gutman 1987, Eisen 1999, Hancock 1999, Zane *et al.* 2002).

In UCO, the two chromosome strands are misaligned during crossing-over, which results in a deletion in one DNA molecule and an insertion in the other. This happens most easily for tandem repeated sequences where the recombination machinery cannot easily determine the correct annealing between two strands (Hancock, 1999). In slipped-strand mispairing, during replication the nascent strand may re-anneal out of phase with the template strand. Eventually, the nascent strand will be longer than the template if the looped-out bases occurred in the nascent strand (insertion mutation) or shorter if the looped-out bases occurred in the template strand (deletion mutation) (Hancock 1999, Brohede 2003) (Figure 2.5).